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Review

Iron–sulfur cluster biogenesis in mammalian cells: New insights into the molecular mechanisms of cluster delivery ☆

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ABSTRACT

Iron–sulfur (Fe–S) clusters are ancient, ubiquitous cofactors composed of iron and inorganic sulfur. The combination of the chemical reactivity of iron and sulfur, together with many variations of cluster composition, oxidation states and protein environments, enables Fe–S clusters to participate in numerous biological processes. Fe–S clusters are essential to redox catalysis in nitrogen fixation, mitochondrial respiration and photosynthesis, to regulatory sensing in key metabolic pathways (*i.e.* cellular iron homeostasis and oxidative stress response), and to the replication and maintenance of the nuclear genome.

Fe–S cluster biogenesis is a multistep process that involves a complex sequence of catalyzed protein–protein interactions and coupled conformational changes between the components of several dedicated multimeric complexes. Intensive studies of the assembly process have clarified key points in the biogenesis of Fe–S proteins. However several critical questions still remain, such as: what is the role of frataxin? Why do some defects of Fe–S cluster biogenesis cause mitochondrial iron overload? How are specific Fe–S recipient proteins recognized in the process of Fe–S transfer?

This review focuses on the basic steps of Fe–S cluster biogenesis, drawing attention to recent advances achieved on the identification of molecular features that guide selection of specific subsets of nascent Fe–S recipients by the cochaperone HSC20. Additionally, it outlines the distinctive phenotypes of human diseases due to mutations in the components of the basic pathway. This article is part of a Special Issue entitled: Fe/S proteins: Analysis, structure, function, biogenesis and diseases.

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1. Introduction

First identified around 1960 in studies of photosynthetic organisms [1] and nitrogen-fixing bacteria [2], and later in succinate dehydrogenase enzymes of porcine and bovine origins [3,4], Fe–S clusters are prosthetic groups common to the most ancient components of living matter. Ferredoxins were among the first representatives of the Fe–S metalloprotein family to be identified and have their iron content and their redox properties characterized [3]. By the mid-1960s, additional Fe–S proteins of bacterial origin were already the subject of intense studies, and they were shown to contain complexes of iron and acid-labile sulfide in the form of two- and four-iron clusters [3]. It quickly became evident that Fe–S species of nuclearities 1, 2, and 4 could be synthesized in aprotic anaerobic solvents in a cell-free system, and these synthetic Fe–S clusters served as accurate analogs of the protein-bound centers [5,6]. The widespread occurrence of Fe–S proteins and their particular abundance in hyper-thermophilic archeabacteria living at around 100 °C in deep oceanic faults support the notion that Fe–S centers were already present as inorganic metal compounds in the anaerobic atmosphere of earth, more than 4.2 billion years ago [7], and that they might have been incorporated into early metabolic pathways by primitive organisms [8]. An important role for Fe–S clusters in the origin of life explains the high level of conservation of Fe–S proteins and of the Fe–S biogenesis machinery among all kingdoms of life: moreover, molecular mechanisms aimed at protecting and enshrouding Fe–S centers during most of the biogenesis and transfer processes likely evolved once plants oxygenated the atmosphere and exposed Fe–S clusters to potential oxidative damage [9]. The vulnerability of Fe–S clusters to oxidation appears to be their Achilles' heel, yet it is also the property that enables them to function as redox active centers for electron transfer in the respiratory chain complexes I to III or in photosystem I of photosynthetic organisms. The bacterial regulatory proteins fumarate and nitrate reduction protein, FNR, and IscR function as O_2 sensors by virtue of their oxygen-sensitive Fe–S clusters [10,11]. Importantly, Fe–S proteins play crucial roles in nitrogen fixation (in nitrogenases) as well as in reduction of protons into hydrogen (in hydrogenases).

Due to their structural versatility, Fe–S proteins are also involved in numerous other functions. A solvent exposed Fe–S cluster directly participates in substrate binding and acid–base catalysis in a family of

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dehydratases, which includes the citric acid cycle enzyme, aconitase. The cytosolic isoform of aconitase is a bifunctional enzyme (iron regulatory protein 1, IRP1/cytosolic aconitase, ACO1), endowed with a crucial function in regulation of cytosolic iron homeostasis [12–14]. Several nucleic acid processing enzymes, such as glycosylases, helicases and primases require Fe–S clusters for their function [15]. Proteins involved in DNA replication have Fe–S centers that are essential for replisome assembly and stability [16]. Moreover, Fe–S clusters are present in DNA repair enzymes (Fanconi anemia group J protein, FANCJ), and in the transcription-coupled nucleotide excision repair (Xeroderma pigmentosum group D protein, XPD) [16,17]. The first Fe–S protein identified as essential for eukaryotic cell viability was the multifunctional ABC-family protein, termed Rli1 in the yeast model, or ABCE1 in humans and other organisms [18,19]. Rli1 has roles in ribosome biogenesis and maturation [18,19], translation initiation [20,21], translation termination [22,23], and ribosome recycling [23]. Integrity of the N-terminal [Fe_4–S_4]-cluster domain of Rli1p is crucial for its function in protein synthesis [24]. S-adenosyl-L-methionine (SAM)-dependent enzymes, such as lipoic acid synthase (LIAS), which is involved in the endogenous synthesis of lipoate [25], and biotin synthase, which is involved in biotin synthesis in lower organisms [26], are also Fe–S proteins.

The remarkable structural versatility of Fe–S clusters constitutes the basis for their widespread occurrence and multiplicity of function. Most frequently, Fe–S proteins contain the rhomboid [Fe_2–S_2], the cuboidal [Fe_3–S_4], and the cubane [Fe_4–S_4] clusters [6]: however more complicated forms have been characterized that also harbor other metal ions [6]. Fe–S clusters are typically ligated by cysteine residues of the polypeptide chain, but other amino acids, such as serine, arginine, and histidine, may also function as ligands. For instance two cysteines and two histidines coordinate the [Fe_2–S_2] cluster of the Rieske protein of respiratory chain complex III.

Although as early as the 1970s it was known that Fe–S clusters could be synthesized from inorganic components *in vitro* under strictly anaerobic conditions [6], in the late 1980s the pioneering work by Dean and co-workers in bacteria revealed that biogenesis of Fe–S clusters *in vivo* is a multistep process that involves a complicated sequence of catalyzed protein–protein interactions and coupled conformational changes between the components of several dedicated multimeric complexes [27,28]. In eukaryotes the scenario is complicated by the requirement for Fe–S proteins in distinct subcellular compartments, including mitochondria, plastids, cytosol and nuclei [29–31]. In mammalian cells, Fe–S clusters are assembled upon the scaffold protein ISCU, through a coordinated set of reactions accomplished by a core complex composed of a cysteine desulfurase, NFS1, its binding partner, ISD11 (LYRM4), and an iron donor or allosteric effector, frataxin [30]. NFS1 abstracts sulfur from free cysteine, and the subsequent reduction of sulfane sulfur (S^0) to sulfide (S^{2-}) likely involves the concerted action of ferredoxin (FDX1 and FDX2 in humans [32]) and ferredoxin reductase (FDXR), which have been shown to provide electrons in prokaryotes [33]. After a nascent cluster forms upon ISCU, it has to be transferred to recipient proteins. A specialized chaperone/cochaperone system has evolved in bacteria (HscA/HscB) and in certain fungi (Ssq1/Jac1), whereas most eukaryotes employ the multifunctional mitochondrial HSP70 (mtHSP70) together with a specialized cochaperone, the orthologue of HscB/Jac1 [34,35].

Intensive studies of the assembly process have clarified key points in the biogenesis of Fe–S proteins. However several critical questions still remain unanswered, including elucidating the role of frataxin, and identifying the molecular mechanisms that guide delivery of Fe–S clusters to specific recipients. Interestingly, studies of several newly described syndromes caused by mutations in NFU1 or BOLA3, and IBA57 (see Table 1) suggest that transfer of Fe–S clusters downstream of the ISCU–chaperone–cochaperone complex depends on selective pathways, which underscores our lack of knowledge about how discrete subsets of Fe–S proteins are targeted for acquisition of their prosthetic groups [30,36].

In this review, we will focus first on the basic steps of Fe–S cluster biogenesis in mammalian cells. We will then discuss recent advances achieved in the identification of molecular features that guide selection of specific Fe–S recipient proteins by the cochaperone HSC20 [37]. Finally, we will outline the distinctive phenotypes of several human diseases that result from mutations in the components of the basic pathway.

2. Fe–S cluster biogenesis: the assembly step

Three distinctive systems, which are required for intracellular formation of Fe–S clusters, have been identified in bacteria, and they are encoded by the *nif*, *isc* and *suf* operons (for a review, see [38]). The *nif*-specific genes in *Azotobacter vinelandii* are involved in biogenesis of Fe–S clusters for nitrogenase. The *Escherichia coli isc*- and *suf*- gene cluster components perform the general synthesis of Fe–S proteins under normal or oxidative stress conditions, respectively [39]. Orthologs of the bacterial *isc* genes have been identified in yeast, plants and animals [40,41], whereas the *suf* system is present mainly in cyanobacteria and plants [40,42]. Particularly intense studies in bacteria and yeast have provided most of the insights into the mechanism of Fe–S cluster assembly (reviewed in [40,41]), revealing that many of the general steps of the pathway are common to all kingdoms of life.

Central to the initial stage of Fe–S cluster biogenesis is a cysteine desulfurase (NFS1 in mammals, Nfs1 in yeast, and IscS in bacteria), which, aided by its cofactor pyridoxal phosphate (PLP), abstracts sulfur from free cysteine and delivers it to a dedicated scaffold protein, ISCU, which provides the cysteine ligands to coordinate the nascent cluster [38] (Fig. 1). The X-ray crystallographic structure of the *E. coli* apo-IscU–IscS complex shows that each IscU molecule interacts with one subunit of the IscS dimer, leading to a 2:2 stoichiometry [43], whereas, to date, no eukaryotic structures of the core complex are available. Using a co-expression approach, Colin and colleagues provided a biochemical and biophysical characterization of the mammalian recombinant ternary complex (named CplxIII) formed by NFS1, ISD11 and ISCU, and of the quaternary complex (CplxIV) that forms when frataxin is added, in the presence of two key substrates, iron and cysteine [44]. The mass of the CplxIII, as determined by electrospray ionization time of flight (ESI-TOF) mass spectrometry, suggested formation of a homodimeric complex in which each unit has a 1:2:1 NFS1:ISD11:ISCU stoichiometry. Frataxin, which was shown to interact with the preformed ternary complex [45], was proposed to bind in a ratio of 1:2:1:1 in the CplxIV complex (NFS1:ISD11:ISCU:FXN) [44].

The cysteine desulfurase activity of the yeast Nfs1 depends on binding to Isd11 [46], a protein not found in prokaryotes [47,48], and recent studies in mammalian cells indicate that the human ortholog LYRM4 (also known as ISD11) is critical for NFS1 activity [49]. Isd11 is present in the mitochondrial matrix of *Saccharomyces cerevisiae*, but it has been detected in both the mitochondrial matrix and the nucleus of mammalian cells [50], supporting previous reports that Fe–S cluster biogenesis components, such as human NFS1 [51], ISCU [52], and the late acting scaffold or specific carrier NFU1 [53], are also present and functional in the cytosolic and/or nuclear compartments of mammalian cells [54].

The source of iron for the nascent cluster remains to be identified, but proposed donors include frataxin [55], which has acidic surface patches to which iron binds with low affinity, or a complex of glutathione and glutaredoxin [56]. Structural modeling studies have recently suggested that frataxin (Yfh1 in yeast) may function as an allosteric effector to either enhance, stabilize [44,45,57], or repress [58,59] the activity of the biogenesis core complex, by interacting with NFS1 and ISCU (Fig. 1). The yeast frataxin ortholog (Yfh1), independently from Isu1 or Isd11, specifically stimulates binding of the substrate cysteine to Nfs1, by inducing exposure of substrate-binding sites, which would be otherwise buried [60]. Importantly, frataxin can compensate for loss of Yfh1 in yeast, indicating that the human protein may act *via* a pathway similar to the one identified in yeast [61,62].

Table 1
Diseases caused by defects in Fe–S cluster biogenesis.

Affected step	Human protein	Yeast ortholog	Putative function(s)	Disease	Cause	Incidence	Tissue(s) affected	References
Core [Fe–S] assembly	NFS1	Nfs1	Cysteine desulfurase—supplies inorganic sulfur	Infantile complex II/III deficiency (IMC23D)	Missense mutation c.215G>A, p.Arg72Gln	Three known patients	Multisystem organ failure	Farhan et al. [216]
Core [Fe–S] assembly	ISD11 (LYRM4)	Isd11	Structural component of the core complex, required for NFS1 activity	Combined oxidative phosphorylation defects	Missense mutation c.203G>T, p.R68L	Two patients known	Skeletal muscle and liver	Lim et al. [49]
Core [Fe–S] assembly	Frataxin (FXN)	Yfh1	Component of the core complex-allosteric effector	Friedreich's ataxia (FRDA)	Heterochromatin dependent repression of *FXN* gene, due to expansion of the intronic GAA repeat. About 4% of patients are compound heterozygotes for the GAA expansion on one *FXN* allele and an inactivating mutation on the second one	1/50,000 births	Dorsal root ganglia, cerebellum and heart	Parkinson et al. [329]; Puccio et al. [330]; Stemmler et al. [55]
Core [Fe–S] assembly	ISCU	Isu1 and Isu2	Primary scaffold for Fe–S cluster assembly	ISCU myopathy	Splicing defect IVS5 + 382G>C, or heterozygosity for the splicing defect and the missense mutation c.149G>A, p.G50E	Approximately 25 patients known	Skeletal muscle. Combination of splicing defect and missense mutation affects heart	Kollberg et al. [231]; Mochel et al. [227]; Olsson et al. [229]
Core [Fe–S] assembly	FDX2	Yah1	Electron donor for Fe–S biogenesis	Mitochondrial muscle myopathy	Disruption of the ATG translation initiation site due to the homozygous mutation c.1A>T	Only one patient known	Skeletal muscle	Spiegel et al. [251]
[Fe–S] transfer to specific recipients	GLRX5	Grx5	Fe–S carrier protein	Sideroblastic anemia	Splicing defect	One patient	Red blood cells	Camaschella et al. [109]; Ye et al. [108]
				Non ketotic hyperglycinemia with childhood-onset spastic paraplegia, spinal lesion, and optic atrophy	Homozygous deletion c.151_153delAAG, p.K51del or compound heterozygosity for p.K51del and 8 bp insertion c.82_83insGCGTGCGG, resulting in premature termination at amino acid 52	Three patients	Primarily affects spinal cord and optic nerve	Baker II et al. [256]
[Fe–S] transfer to specific recipients	NFU1	Nfu1	Fe–S delivery to specific recipients (LIAS)	Multiple Mitochondrial Dysfunctions Syndrome 1 (MMDS1)	c.545G>A substitution near the splice donor site of exon 6 in NFU1 predicting a p.Arg182Gln substitution. Homozygosity for c.622G>T (p.Gly208Cys), or compound heterozygosity for c.622G>T and the splice site c.545 + 5G>A	One patient for c.545G>A. Nine patients for c.622G>T	Early onset severe systemic illness — No tissue specificity observed	Cameron et al. [133]; Navarro-Sastre et al. [135]
[Fe–S] transfer to specific recipients	BOLA3	Aim1	Fe–S delivery to specific recipients (LIAS)	Multiple Mitochondrial Dysfunctions Syndrome 2 (MMDS2)	Single base-pair duplication c.123dupA, in exon 2, causing a frameshift that produces a premature stop codon (p.Glu42Argfs*13)	One patient	Central nervous system and heart	Cameron et al. [133]
					Homozygous missense mutation c.200T>A in exon 3, p.I67N	Two patients	Multisystem organ failure	Haack et al. [134]
				Non ketotic hyperglycinemia with severe neurodegeneration, leukodystrophy, cardiomyopathy and optic atrophy	Homozygosity for c.136C>T, p.R46X truncation	Three patients	Primarily affects central nervous system	Baker II et al. [256]
[Fe_4–S_4] assembly	IBA57	Iba57	[Fe_4–S_4] assembly component for a subset of recipients	Multiple Mitochondrial Dysfunctions Syndrome 3 (MMDS3)	Homozygous mutation c.941A>C, p.Gln314Pro	Two patients	Central nervous system/skeletal muscle	Ajit Bolar et al. [36]
Mitochondrial translation; complex I assembly	NUBPL	Ind1	Mitochondrial translation; complex I assembly	Childhood-onset mitochondrial encephalopathy and complex I deficiency	c.166G>A, p.G56R substitution in NUBPL in combination with deletion spanning exons 1–4. A second substitution was found in intron 9 (c.815-27T>C), resulting in aberrant splicing	At least seven patients identified	Central nervous system, skeletal muscle	Calvo et al. [146]; Wydro and Balk [247]
Mitochondrial export	ABCB7	Atm1	Component of the mitochondrial export machinery	X-linked sideroblastic anemia with cerebellar ataxia (XLSA/A)	Several mutations close to or in transmembrane domains of the ABC transporter	At least twenty-three patients identified	Central nervous system	Allikmets et al. [273]; Bekri et al. [274]; D'Hooghe et al. [294]; Maguire et al. [275]
Mitochondrial iron import	Mitoferrin1 (MFRN1)	MRS3 and MRS4	Mitochondrial iron importer	Variant erythropoietic protoporphyria	Aberrant splicing of *MFRN1* transcript together with mutations in *FECH* or *ALAS2*	Seven patients identified	Erythroblastic cells	Lecha et al. [296]; Wang et al. [309]

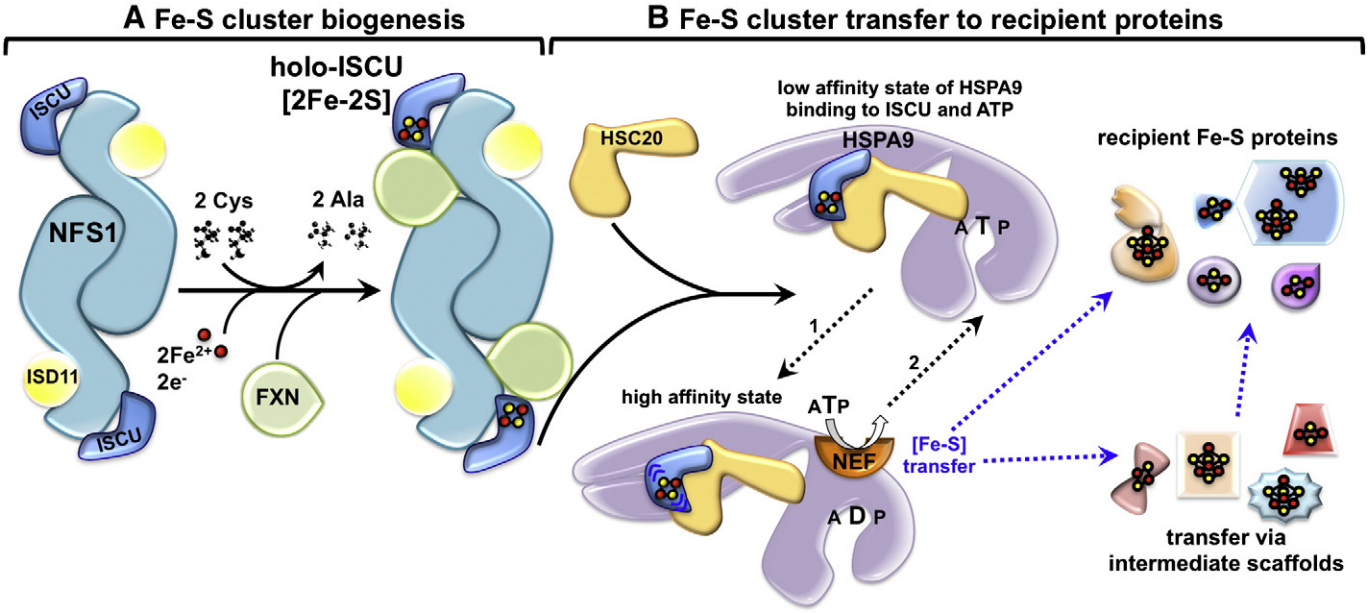

Fig. 1. Fe–S cluster biogenesis in mammalian cells: a general scheme of the main steps. (A) Assembly of a nascent Fe–S cluster upon the scaffold protein ISCU. A cysteine desulfurase NFS1 forms a dimer to which monomers of the primary scaffold ISCU bind at either end. ISD11 is a structural component of the core complex in eukaryotes, required for the activity of NFS1, which, aided by its cofactor pyridoxal phosphate, provides sulfur, removed from cysteine, for the nascent cluster. Frataxin (FXN) is part of the core complex, potentially binding in a pocket-like region between NFS1 and ISCU. The cluster assembles upon ISCU when iron is provided together with the reducing equivalents that allow achievement of the final electronic configuration of the cluster. (B) Cluster transfer to recipient apoproteins. A dedicated chaperone/cochaperone (HSPA9/HSC20) system facilitates cluster transfer from the primary scaffold ISCU to recipient apoproteins or to intermediate carriers, which then target specific recipients. The cochaperone HSC20 interacts with ISCU through a patch of hydrophobic amino acid residues in the C-terminus, and with the chaperone HSPA9 through the N-terminal domain (J-domain). The J-domain of HSC20 contains a conserved His (H), Pro (P), Asp (D) tripeptide essential for activation of the ATPase activity of HSPA9. The energy derived from activation of HSPA9 drives a conformational rearrangement in the substrate-binding domain of the chaperone (see text for further details), which is proposed to propagate a conformational change to its binding partner, ISCU (arrow 1). The conformational change is thought to facilitate release of the cluster from the main scaffold, and allow protected transfer directly or *via* intermediate carriers to specific subsets of Fe–S recipient proteins. A nucleotide exchange factor (NEF), known as Mge1 in yeast and SIL1 (or BAP) in humans exchanges ADP with ATP and completes the ATPase cycle of the chaperone (arrow 2).

Electrons, needed to achieve the final electronic configurations of Fe–S clusters, are provided by ferredoxins (FDX1/2) and ferredoxin reductase (FDXR) [32,63]. Ferredoxin has also been shown to facilitate reductive coupling of two distinct [Fe_2–S_2] clusters into a single [Fe_4–S_4] cluster on bacterial IscU *in vitro* [64].

3. Transfer of Fe–S clusters to recipient proteins

In vivo studies using a dominant negative mutant of the bacterial scaffold IscU ($IscU^{D39A}$ in *A. vinelandii*, or $IscU^{D35A}$ in *Archaeglobus fulgidus*), which remains trapped in a complex with the sulfur donor IscS, and contains a nascent Fe–S cluster [65,66], showed that the cluster assembly and release steps could be uncoupled. For the Fe–S cluster release and transfer process, bacterial IscU interacts with two dedicated partners, HscA and HscB, members of the DnaK/DnaJ (chaperone/cochaperone) families, respectively [67]. The Fe–S cluster transfer machinery is highly conserved among different organisms [40]. A cochaperone known as HSC20 in mammalian cells [68] (HscB in bacteria [34], and Jac1 in *S. cerevisiae* [69]) binds to the scaffold protein ISCU, and forms a complex with its chaperone partner, HSPA9 (HscA in bacteria, and Ssq1 in *S. cerevisiae*), a member of the HSP70 heat shock protein family [37,70,71]. HSP70 homologues use the energy released by hydrolysis of ATP to drive conformational changes and refolding of target proteins [72]. Both in the bacterial and mitochondrial systems, the C-terminal domain of the cochaperone is directly responsible for binding the scaffold protein ISCU, with three highly conserved hydrophobic residues being of crucial importance for the HSC20–ISCU interaction [37,73–75]. The crystal structures of HscB from *E. coli* [76], of Jac1 from *S. cerevisiae* [74], and of human HSC20 [77] revealed common features among cochaperones dedicated to Fe–S cluster biogenesis (Fig. 2). They have a conserved structural core that consists of two domains arranged in a L-shaped fold. The N-terminal J-domain, which contains an invariant histidine, proline, aspartate (HPD) motif, is responsible for stimulating the ATPase activity of its HSP70 cognate chaperone [37,76,78,79], whereas the C-terminal domain forms a three-helix bundle. The N-terminus of human HSC20 (Fig. 2A) is clearly different from the specialized DnaJ type III cochaperones of bacteria and fungi, in that it contains, adjacent to the mitochondrial targeting sequence (residues 1–26 [70]), an extra-domain, which harbors two CxxC modules (C41/C44 and C58/C61) of unknown function (Fig. 2A). Their ability to coordinate a zinc ion *in vitro* results in a zinc finger-like structure [77].

Recent studies in yeast indicate that the scaffold protein Isu1 binds to the cysteine desulfurase Nfs1 or to the cochaperone, Jac1, in a mutually exclusive fashion [80]. Three highly conserved surface-exposed hydrophobic residues of Isu1 (L63, V72 and F94) were found to be critical for the interaction with hydrophobic residues of Jac1 (L109, L105, and Y163, respectively), or with residues of Nfs1 (M482, P478, and L479, respectively), thus suggesting a functional role for the mutual exclusivity of these interactions: competition between Jac1 and Nfs1 for binding to Isu1 may play an important role in transitioning the Fe–S cluster biogenesis process from the core complex, which accomplishes the initial assembly step, to the chaperone–cochaperone system that facilitates transfer of the cluster to recipient proteins [80]. Evolutionary analyses revealed that residues involved in these interactions are highly conserved, thus suggesting that competition for binding to Isu1 is a point of regulation between initial assembly and transfer of the nascent cluster to recipient proteins that might be conserved from bacteria to humans. Cochaperones serve a dual function in Fe–S cluster biogenesis: they escort ISCU to the substrate binding domain of the HSP70 cognate chaperone, and stimulate the ATPase activity of the chaperone, perhaps driving a conformational change that could facilitate cluster release from ISCU and delivery to the final acceptor apoprotein or to intermediate carriers, which ultimately donate their clusters to specific recipients

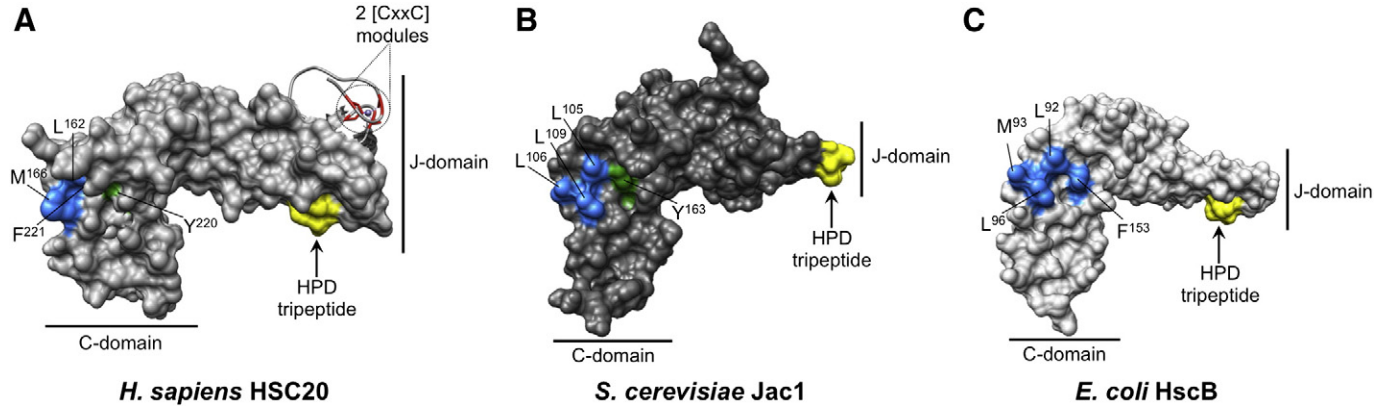

Fig. 2. 3D-structures of cochaperones dedicated to Fe–S cluster biogenesis in *H. sapiens* (HSC20, panel A), *S. cerevisiae* (Jac1, panel B), and *E. coli* (HscB, panel C). Conserved amino acid residues which interact with the main scaffold ISCU on the surface of the C-terminal domains of the cochaperones dedicated to Fe–S cluster biogenesis in humans (A), yeast (B), and bacteria (C): in blue are the hydrophobic amino acids (Leu, Met, and Phe), and in green the polar residues (Tyr). The His (H), Pro (P), Asp (D) tripeptide in the N-terminal domains (J-domains) of the cochaperones is indicated by arrows and colored in yellow. Two CxxC modules, which coordinate zinc in the crystal structure of human HSC20, are shown in red.

[34,81] (Fig. 1). Studies in bacteria and yeast have defined the residues of the IscU scaffold protein which mediate the interaction with the HSP70 chaperone [34,82], and the crystal structure of the substrate binding domain of HscA in complex with a peptide containing the IscU recognition region (LPPVK) has been determined [83]. The crystal structure of the substrate-binding domain of HSPA9 (residues 439–590) is also available (PDB ID: 3N8E), and it shares 97% similarity to the domain of bacterial HscA that binds IscU. The high degree of sequence homology translates into a remarkable structural conservation of the domains of the two proteins (Fig. 3A–C).

3.1. Determining specificity of Fe–S cluster delivery: molecular features that guide selection of recipient Fe–S proteins

A long-standing question in the field has been related to the molecular mechanisms that guide selection of specific Fe–S recipient proteins by the chaperone–cochaperone system. Most eukaryotes, including humans, have a single multifunctional mitochondrial chaperone, which participates in diverse cellular functions by virtue of its ability to interact with an array of different J proteins (also known as HSP40s) (for reviews, see [72,84,85]). HSP70s have a promiscuous ATP-dependent substrate binding recognition activity. Cochaperones are thought to play the crucial role of driving the functional specificity of HSP70s [35,72,74], mostly through their C-terminal domains, which can bind substrates on their own [86,87], facilitate refolding of denatured proteins, and enhance cell viability [88,89]. HSC20 is the sole human DnaJ type III cochaperone dedicated to Fe–S cluster biogenesis [68]. Mutations in HSC20 and in its orthologs cause defects in Fe–S protein activities, mitochondrial iron accumulation, and defective mitochondrial oxidative phosphorylation in human cell lines [68], and in multiple experimental systems, including yeast [79,90] and fly [91].

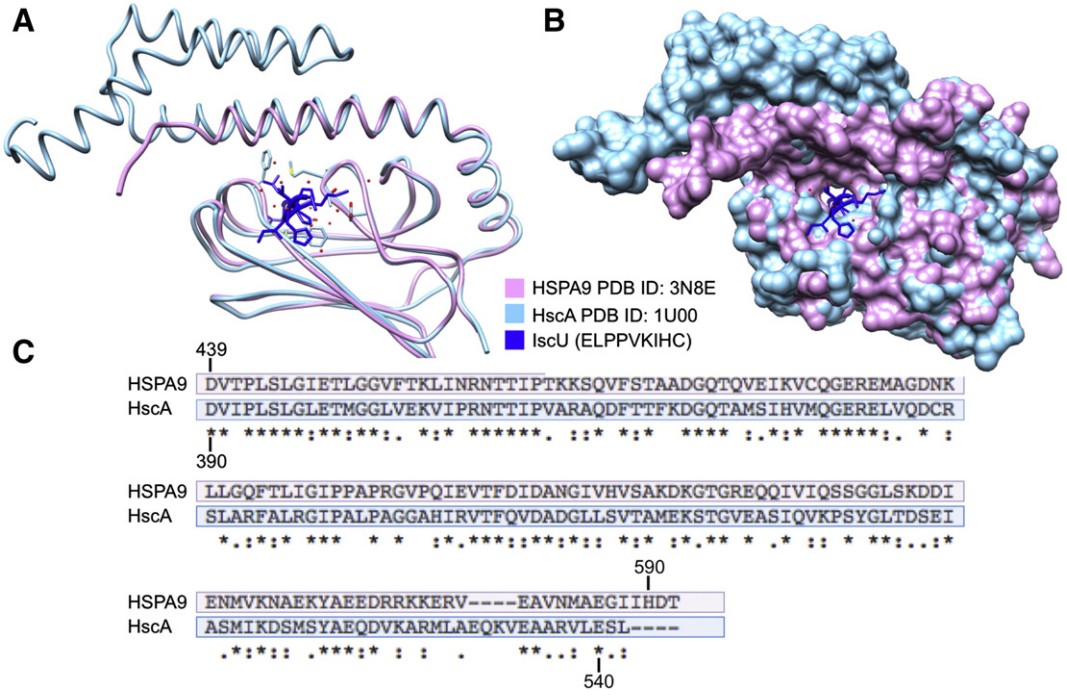

Fig. 3. 3D-structural comparison between the substrate-binding domains of bacterial HscA and human HSPA9, chaperones involved in Fe–S cluster biogenesis. Three-dimensional comparison of the crystal structures of the substrate-binding domain of HscA (residues 390–540, in cyan) in complex with a peptide containing the IscU recognition region (LPPVK in blue), and the region 439–590 of human HSPA9 (in violet). Panels A and B. Ribbon representation (A) and equivalent surface representation (B) of the two overlapped structures of the substrate binding domains of HscA and HSPA9. (C) Primary sequences of the regions of the two HSP70s showed in (A) and (B).

Recent studies have shown that HSC20 guides selection of Fe–S cluster delivery by binding to a conserved leucine–tyrosine–arginine (LYR) motif present in specific recipient Fe–S proteins or in accessory factors that likely assist Fe–S cluster insertion into target apoproteins [37]. Several HSC20 binding partners were identified in a stringent yeast two-hybrid (Y2H) screen, a method of identifying direct molecular interactions between pairs of proteins, and some of them were validated *in vivo* by co-immunoprecipitation studies. Notably, multiple individual clones identified in the screen encoded succinate dehydrogenase subunit b (SDHB), the Fe–S cluster containing subunit of complex II, which has three Fe–S centers of different nuclearities. To identify the potential motifs in SDHB that could bind HSC20, the SDHB primary sequence was subdivided into multiple peptides, which were cloned into Y2H prey constructs to screen 50 to 100 subclones of varying sizes for their ability to interact with HSC20. Three independent binding sites for HSC20 were identified on SDHB, and two of these were iterations of the LYR motif. The LYR motif was present in several other proteins selected in the Y2H (*i.e.* EPRS, HELZ, SPC25, GTF2E2, ETFA), and also in LYRM7, the Rieske Fe–S protein chaperone of complex III [92] and an annotated member of the LYR family [93] (Conserved Domains Accession: cl05087). The LYR motif family contains at least eleven proteins in humans in which the LYR tripeptide resides close to their N-terminus. Members of the LYR family are SDHAF1 (LYRM8), a *bona fide* succinate dehydrogenase complex assembly factor [94], ISD11 (LYRM4), a component of the initial Fe–S assembly complex [50], LYRM7, a complex III assembly factor, and two subunits of respiratory chain complex I known as NDUFA6 and NDUFB9 (also annotated as LYRM6 and LYRM3, respectively) [93]. Several LYR motif proteins do not have a known function, such as LYRM2, LYRM5, and LYRM9, whereas others have been only partially characterized in yeast, such as ACN9 (acetate non-utilizing protein 9) [95,96], or FMC1 (formation of mitochondrial complexes protein 1) [97]. Interestingly, human ISD11 and LYRM7 share high sequence homology with their yeast orthologues, Isd11 and Mzm1, respectively, and they perform similar functions [50,92], whereas human SDHAF1 has low similarity to the yeast ortholog Sdh6 and failed to restore respiration in a *Sdh6* null background (Δ*Sdh6*) yeast strain [94]. The functional significance of the common LYR motif was completely unknown when the Pfam clan Complex1_LYR-like superfamily (CL0491) was built by P.C. Coggill [98]. Based on bioinformatics analyses, the motif was defined as a tripeptide in which the first position was generally an aliphatic hydrophobic amino acid such as isoleucine or leucine, the second was an aromatic amino acid such as tyrosine or phenylalanine, and the third was a positively charged arginine or lysine. The tripeptide was followed by a conserved phenylalanine separated from LYR by about twenty-five residues [93]. The coimmunoprecipitation and Y2H studies indicated that LYR motifs present in known recipient Fe–S proteins (*i.e.* SDHB) or in accessory factors (*i.e.* SDHAF1, LYRM7) mediated an interaction with the cochaperone HSC20, and likely guided insertion of Fe–S clusters into the Fe–S subunit of respiratory chain complex II (Fig. 4), and, possibly, into complex III [37]. In fact, LYRM7, which has been characterized as a complex III assembly factor, binds UQCRFS1 [92], the Rieske Fe–S protein of complex III, and engages the HSC20–HSPA9–ISCU complex [37], suggesting that the LYRM7–HSC20 interaction might guide the insertion of the [Fe_2–S_2] cluster into UQCRFS1. The role of the complex I subunit LYRM6 (also known as NDUFA6 and as NB4M in the yeast *Yarrowia lipolytica*) has been recently characterized [99]. Chromosomal deletion of NB4M in *Y. lipolytica* or mutagenesis of the LYR tripeptide and of a conserved downstream phenylalanine into alanines caused loss of the [Fe_4–S_4] N2 cluster, which is the last Fe–S center in the ascending electron transfer sequence of the matrix arm of complex I with the highest redox potential, and totally abrogated the ubiquinone reductase activity of complex I, despite the fact that all the central subunits of the complex were intact. This finding suggests that NB4M (NDUFA6) is required for proper incorporation of the N2 [Fe_4–S_4] center, and for the function of complex I. Notably, NDUFS8, which ligates two [Fe_4–S_4] clusters, contains a conserved LYR motif in a non-canonical position, at its C-terminus. As only 14 of the 45 complex I subunits have a catalytic function [100], some of the accessory or supernumerary subunits may contribute to the assembly and stability of the complex, and the LYR-containing subunits may aid insertion of some of the Fe–S clusters.

A second consensus sequence, $KKx_{6-10}KK$, was identified in the screening of the SDHB peptides that interacted with HSC20 through its C-terminal domain. Interestingly, human glutaredoxin 5 (GLRX5) has a similar pattern of lysines at its C-terminus ($K_{139}K_{140}x_{10}K_{151}K_{151}$) and was found to interact with HSC20 *in vivo* [37]. The yeast ortholog Grx5 interacts with Ssq1, the mitochondrial chaperone dedicated to Fe–S cluster biogenesis [101].

The function of the LYR and $KKx_{6-10}KK$ motifs may be very dependent on their molecular context and structural location [37]. Other features may define which LYR proteins engage HSC20 and the transfer apparatus, and these features will likely be defined as more LYR and Fe–S proteins are studied.

3.2. Intermediate carriers and secondary scaffolds that deliver Fe–S clusters to recipient proteins

Glutaredoxin 5 has been characterized as one of many intermediate carriers which are involved in the transfer of Fe–S clusters downstream of the scaffold protein ISCU, in yeast [41,101,102], zebrafish [103], and humans [104–109]. In eukaryotes, monothiol glutaredoxins localize to mitochondria or chloroplasts and have been implicated in Fe–S cluster biogenesis (for recent reviews, see references [104,110–112]), whereas multidomain Glrxs (*i.e.* yeast Grx3/4 and human GLRX3) localize to the cytosolic/nuclear compartment and are proposed to play dual roles in iron trafficking and regulation [113–116]. The first crystal structure of a [Fe_2–S_2]-bridged Grx homodimer, published in 2009 for *E. coli* Grx4, showed that two GSH molecules were held in place by non covalent interactions with the binding pocket of each Grx4 monomer and by covalent linkages to the cluster [117]. A more recent crystal structure of human GLRX5 revealed a similar coordination for the [Fe_2–S_2] cluster; however, in this structure, two [Fe_2–S_2]-bridged homodimers interacted to form a tetramer [118]. The current working model to describe the role of glutaredoxins in Fe–S cluster biogenesis proposes that they transiently accept a [Fe_2–S_2] cluster and engage the chaperone–cochaperone transfer apparatus in order to facilitate its insertion into final acceptor apoproteins [37,101]. An alternative cluster transfer mechanism hypothesizes that a [Fe_2–S_2] cluster coordinated by four glutathione molecules might be exchanged with the cellular Fe–S cluster biogenesis components [119,120]. Glutaredoxins have been linked to another family of widely distributed proteins, the BolA-like proteins, by bioinformatics analyses, yeast two-hybrid and affinity purification studies, both in prokaryotes and in eukaryotes, including plants [121–125]. BolA proteins were initially identified in *E. coli* with the observation that their overexpression promoted a switch from rod-shaped to rounded cell morphologies [126]. The Grx3/Grx4 proteins are involved in iron regulation in yeast, by forming a complex with the BolA-like protein, Fra2, and ligating a [Fe_2–S_2] cluster coordinated by the Grx active site cysteine of Grx3/Grx4, a conserved histidine in Fra2 (His103), and a cysteine from glutathione [114,127,128] (for a review on Grxs and BolA-like proteins, see reference [105]). Under low iron conditions or upon disruption of mitochondrial Fe–S cluster biogenesis, the Fra2/Grx3/Grx4 signaling pathway is proposed to interpret and transmit a signal to the iron-responsive transcription factor Aft1 (and presumably its paralog Aft2), allowing Aft1 to accumulate in the nucleus and activate the iron regulon [114–116], which encodes proteins involved in iron uptake, transport, and storage [129–131]. Under iron-replete conditions, an inhibitory signal is transmitted to Aft1 (and/or Aft2) that induces its multimerization and export from the nucleus *via* its interaction with the exportin Msn5 [132]. Mammals have three BOLA homologues, named BOLA1, BOLA2, and BOLA3, of unknown functions. Recently, mutations in human BOLA3 have been

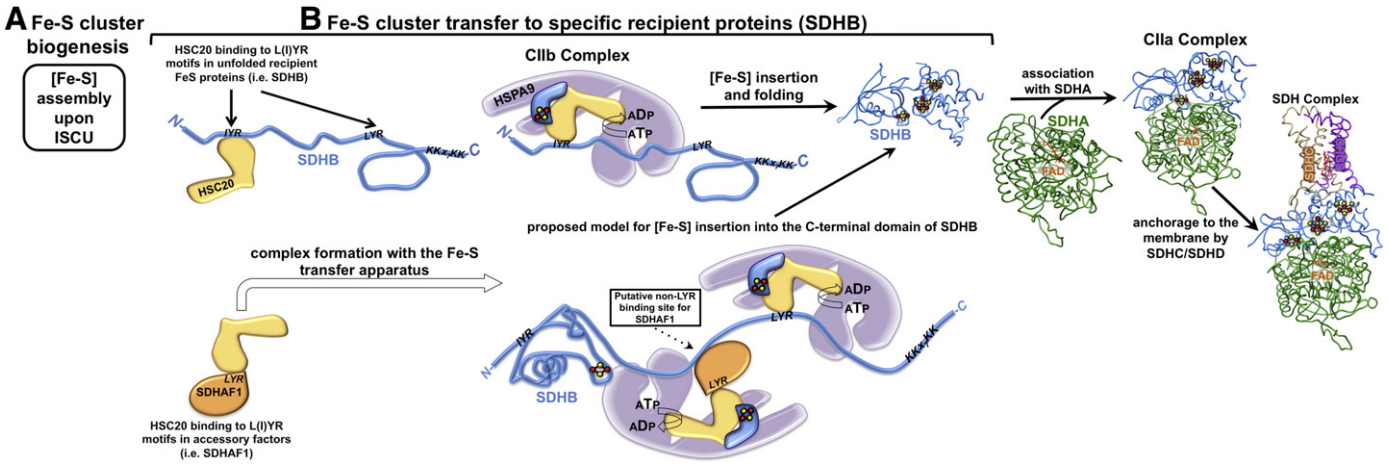

Fig. 4. Schematic representation of Fe–S cluster delivery to specific recipients mediated by binding of HSC20 to LYR motifs. Fe–S cluster incorporation into succinate dehydrogenase B (SDHB): in SDHB, two LYR motifs (IYR and LYR) engage the HSC20–HSPA9–ISCU complex, which assists incorporation of three Fe–S clusters within the final structure of complex II. An important aspect of the model is that the first motif may enter the mitochondrial matrix first, as the SDHB polypeptide translocates from the site of its synthesis in the cytosol, to its destination, the mitochondria. The accessibility of the first motif would not be yet affected by C-terminal sequences and secondary structure formation. Indeed, donation of a [Fe_2–S_2] cluster could determine secondary structure by driving folding around the Fe–S cluster, to which cysteines 93, 98, 101, and 113 bind with high affinity. SDHAF1 (LYRM8), an assembly factor for complex II and an annotated member of the LYR motif family, associates with SDHB through a non-LYR binding site that is not yet fully defined, but that resides between the two LYR motifs. In our working model, SDHAF1 binds to SDHB through a non-LYR portion of its sequence and utilizes its own LYR motif to position an ISCU–HSC20–HSPA9 complex near to Fe–S transfer complexes directly associated with the second LYR binding site of SDHB. The propensity of HSC20 to dimerize may allow two holo-ISCU molecules at neighboring binding sites to reorganize their adjacent [Fe_2–S_2] centers, thus coalescing into the [Fe_4–S_4] and [Fe_3–S_4] clusters of mature SDHB. Again, the primary peptide would be driven to fold around the newly incorporated Fe–S clusters, potentially explaining how some Fe–S centers can be deeply buried in proteins. The structures reported are relative to porcine succinate dehydrogenase complex (96% identical to human. PDB ID: 3SFD).

discovered to cause disease in patients diagnosed with multiple mitochondrial dysfunctions syndrome 2 [133,134], caused by defects in the biogenesis of the Fe–S cluster enzyme lipoic acid synthase (LIAS). Human BOLA3 has two isoforms (NM_001035505.1 and NM_212552.2) that result from alternative splicing, with isoform 2 lacking exon 3, and they are both predicted to localize to mitochondria with 95% probability. Human NFU1, which was shown to accommodate a [Fe_4–S_4] cluster [53], has been recently characterized as a late acting factor specifically required for maturation of lipoic acid synthase, rather than as an alternative scaffold acting in parallel to ISCU, based on the fact that mutations in NFU1 are associated mainly with defective lipoic acid (LA) metabolism [133, 135,136], whereas other Fe–S dependent processes are less affected. The A-type scaffolds are thought to support the biogenesis of a distinct subset of Fe–S proteins. The *isc* and *suf* operons of bacteria encode two A-type proteins IscA and SufA, respectively, which were found to be involved in the maturation of some [Fe_4–S_4] proteins, such as aconitase and LIAS [38,137]. An additional member of this family, ErpA, is specifically involved in isoprenoid biosynthesis [138]. Eukaryotes have two A-type ISC proteins that share common primary structure features, but have different functions [139,140]. Yeast Isa1 and Isa2 form a complex with Iba57, a protein that was initially identified in a genome-wide screen for *S. cerevisiae* mutants that required supplementation of lysine and glutamate for growth [141]. Iba57 was later found to be required for Fe–S cluster incorporation into aconitase and homoaconitase and into the radical-SAM Fe–S enzymes biotin synthase and lipoic acid synthase [141,142]. The human genome encodes two A-type proteins, named ISCA1 and ISCA2, related to *S. cerevisiae* Isa1 and Isa2, respectively, thought to be necessary for biogenesis of several [Fe_4–S_4], but not [Fe_2–S_2], enzymes [143]. Cells deficient in IBA57 exhibited a phenotype similar to ISCA1- or ISCA2-depleted cells. Consistent with the role of ISCA1/ISCA2 and IBA57 in the biogenesis of [Fe_4–S_4] clusters for a subset of mitochondrial proteins, a mutation affecting the stability of IBA57 was recently found in two patients with severe myopathy and encephalopathy associated with compromised activities of the lipoic acid dependent enzymes, indicating defective maturation of LIAS [36]. The mitochondrial P-loop NTPase IND1 (Fe–S protein required for NADH dehydrogenase) was initially thought to be an iron–sulfur protein required for effective complex I assembly in the yeast *Yarrowia lipolitica* [144]. Studies of the human ortholog NUBPL (nucleotide–binding protein–like) similarly suggested that the protein was a specific Fe–S cluster targeting factor for complex I [145]. NUBPL was therefore included in a list of 103 candidate genes for next-generation exon sequencing in patients with complex I deficiency [146]. Recent studies of IND1 in *Arabidopsis thaliana* (INDH) were able to untangle primary from secondary phenotypes caused by inactivating mutations in INDH, and demonstrated that INDH has a primary role in mitochondrial translation [147]. Impairment of complex I likely was due to the fact that seven of its subunits are encoded by the mitochondrial genome and translated in the matrix.

4. Cytosolic iron–sulfur cluster assembly machinery (CIA)

Details of how the mitochondrial and cytosolic Fe–S cluster assembly pathways are connected have been the subject of intense scrutiny, and the paradigm that Fe–S clusters are assembled solely in the mitochondrial matrix based on studies in yeast does not necessarily extrapolate to mammalian cells [30], where extra-mitochondrial isoforms of the core components NFS1 [51,148], ISD11 [50], and ISCU [54], and of the intermediate carrier NFU1 [53], have been identified, together with nine additional proteins, highly conserved from yeast to humans, which constitute the cytosolic iron–sulfur assembly machinery (CIA) [149]. Interestingly, a pool of HSC20 is present in the cytosol of mammalian cells [37,68], and the cochaperone from *A. thaliana* (AtHscB), which is able to rescue the Jac1 yeast knock out mutant, localizes both to the mitochondrial and cytosolic compartments [150], together with the scaffold protein AtIscU1 and the specialized chaperone dedicated to Fe–S cluster biogenesis, AtHscA [150].

An "export system" has been proposed to transport a specific sulfur-containing compound out of the mitochondrial matrix, which can then be utilized for cytosolic Fe–S cluster biogenesis [107]. The ABC transporter ABCB7 (Atm1 in yeast) is part of the putative export machinery, together with the sulfhydryl oxidase GFER (yeast Erv1) [151], and glutathione [152]. Defects in ABCB7/Atm1 cause impaired cytosolic Fe–S cluster protein activities and iron accumulation in mitochondria [153,154]. In yeast, Fe–S cluster biogenesis in the cytosol is proposed to initiate with

the assembly of a nascent [Fe_4–S_4] cluster upon the scaffold complex composed of the two P-loop NTPases Cfd1 and Nbp35 (NUBP2, NUBP1 in humans, respectively — see Table 2) [155]. This proposed initial step also requires transfer of electrons from NADPH to the diflavin reductase Tah18 and the Fe–S protein Dre2 (NDOR1 and CIAPIN1 (anamorsin), respectively, in humans) [156]. The second stage (transfer of the transiently bound [Fe_4–S_4] cluster from Cfd1-Nbp35 to recipient apoproteins) is accomplished by the iron-hydrogenase-like protein Nar1 (also known as NARFL or IOP in humans) [157], and the CIA targeting complex formed of Cia1, Cia2 and Mms19 (CIAO1, CIA2B, CIA2A and MMS19 in humans) [158–161]. MMS19 is part of the late-acting CIA complex, together with CIAO1 and CIA2B, that specifically targets cytosolic Fe–S proteins that are linked to nucleotide and DNA metabolism (*i.e.* glutamine phosphoribosylpyrophosphate amidotransferase (GPAT) and dihydropyrimidine dehydrogenase (DYPD), DNA polymerase δ (Polδ), xeroderma pigmentosum protein D (XPD), the RAD3-like helicases RTEL1 (regulator of telomere length protein 1) and Fanconi anemia protein J (FANCJ)) [159,160]. Conversely, MMS19 is dispensable for the maturation of the Fe–S cluster for cytosolic aconitase (ACO1/IRP1), which is believed to acquire its cluster from a distinct complex formed by CIAO1 and CIA2A [161]. CIAO1 has indeed been shown to form mutually exclusive complexes with either CIA2A or CIA2B–MMS19. The CIAO1–CIA2B–MMS19 complex appears to facilitate maturation of most of the cytosolic–nuclear Fe–S proteins, whereas CIA2A seems to be specifically involved in the biogenesis of Fe–S clusters for cytosolic aconitase, which is converted into the IRE (Iron Responsive Element)-binding form when cells are CIA2A depleted [161]. An additional role of CIA2A in cellular iron regulation may involve stabilization of IRP2 [159,161], which does not contain an Fe–S cluster, but undergoes iron- and oxygen-dependent degradation by the proteasome [162,163].

5. Defects in Fe–S cluster biogenesis and human diseases

As previously discussed, Fe–S clusters are involved in several fundamental cellular processes, and it is therefore not surprising that mutations that affect the components of the basic Fe–S biogenesis pathway cause diseases in humans (see Table 1).

5.1. Friedreich's ataxia (MIM# 229300)

Friedreich's ataxia (FRDA) is an autosomal recessive neurodegenerative disorder caused primarily by a homozygous GAA repeat expansion within intron 1 of the frataxin (*FXN*) gene, located on chromosome 9q21.1 [164]. Approximately 4% of FRDA patients are compound heterozygotes, having a GAA repeat expansion on one allele and an inactivating or loss-of-function mutation, such as a point mutation [165,166] or a deletion/duplication [167–169] on the other allele. The incidence of FRDA is 1–2 in 50,000 in Caucasian populations with an equal occurrence in both genders [170,171] and an estimated carrier frequency of 1:60 to 1:100 [172]. Unaffected individuals have up to 43 GAA repeats, whereas FRDA patients have 44 to 1700, most commonly between 600–900 GAA repeats [172,173]. Genotype/phenotype correlation studies have been carried out to assess the relationship between the length of the GAA-triplet repeat and the clinical manifestations in FRDA [174]. Larger GAA repeat expansions correlate with increased severity of FRDA disease and with earlier age of onset [174,175]. The GAA-triplet repeat leads to transcriptional silencing of *FXN* that is caused most likely by heterochromatin condensation of the locus [176], which drastically reduces transcription of *FXN* by 70%–95% [177,178]. A recent investigation reports on the transcriptional silencing of *FXN*, caused by formation of RNA/DNA hybrids (named R-loops) [179], which are stable and co-localize with repressive

Table 2
Proteins involved in Fe–S cluster biogenesis in eukaryotes.

Assembly step	Human protein	Yeast ortholog	(Putative) function(s)
Core Fe–S cluster assembly	NFS1	Nfs1	Sulfur donor
Core Fe–S cluster assembly	ISCU	Isu1 and Isu2	Scaffold protein
Core Fe–S cluster assembly	ISD11 (LYRM4)	Isd11	Eukaryotic specific accessory factor required for cysteine desulfurase activity
Core Fe–S cluster assembly	Frataxin (FXN)	Yfh1	Iron donor (?) Allosteric effector (?) Cysteine desulfurase derepressor
Core Fe–S cluster assembly	Ferredoxins (FDX1, FDX2)	Yah1	Electron transport
Core Fe–S cluster assembly	Ferredoxin reductase (FDXR)	Arh1	Electron transport
[Fe–S] cluster transfer	Mortalin (HSPA9, GRP75)	Ssq1	Molecular chaperone
[Fe–S] cluster transfer	HSC20	Jac1	Cochaperone. It binds target proteins that contain the LYR motif
[Fe–S] cluster transfer	SIL1 (BAP)	Mge1	Nucleotide exchange factor
[Fe–S] cluster transfer	GLRX5	Grx5	Intermediate carrier
[Fe–S] cluster transfer-late acting factor	ISCA1	Isa1	[Fe_4–S_4] assembly
[Fe–S] cluster transfer-late acting factor	ISCA2	Isa2	[Fe_4–S_4] assembly
[Fe–S] cluster transfer-late acting factor	IBA57	Iba57	[Fe_4–S_4] assembly
[Fe–S] cluster transfer-late acting factor	NFU1	Nfu1	Dedicated targeting factor for LIAS
[Fe–S] cluster transfer-late acting factor	BOLA3	Aim1	Dedicated targeting factor for LIAS
[Fe–S] cluster transfer-late acting factor	NUBPL	Ind1	Mitochondrial translation; complex I assembly
Mitochondrial export of an unknown compound to the cytosol	ABCB7	Atm1	ABC transporter proposed to export a sulfur containing compound from mitochondria
Mitochondrial export of an unknown compound to the cytosol	GFER	Erv1	FAD-dependent sulfhydryl oxidase in intermembrane space that catalyzes disulfide bond formation. Required for cytosolic Fe–S cluster assembly
Extramitochondrial [Fe–S] biogenesis	NUBP1	Nbp35	P-loop NTPase that forms a heterotetrameric Fe–S scaffold with NUBP2
Extramitochondrial [Fe–S] biogenesis	NUBP2	Cfd1	P-loop NTPase that forms a heterotetrameric Fe–S scaffold with NUBP1
Extramitochondrial [Fe–S] biogenesis	NARFL (IOP)	Nar1	Interacts with CIAO1 and can mediate Fe–S cluster transfer
Extramitochondrial [Fe–S] biogenesis	CIAO1	Cia1	WD40 repeat protein that interacts with NARFL and can mediate Fe–S cluster transfer
Extramitochondrial [Fe–S] biogenesis	NDOR1	Tah18	NADPH-dependent diflavin oxidoreductase 1. Electron transport
Extramitochondrial [Fe–S] biogenesis	CIAPIN1	Dre2	Electron transport
Extramitochondrial [Fe–S] biogenesis	MMS19	MET18	Adapter between early acting CIA components and a subset of cellular target Fe–S proteins
Extramitochondrial [Fe–S] biogenesis	FAM96A (CIA2A)		Part of a complex that facilitates Fe–S cluster transfer to IRP1
Extramitochondrial [Fe–S] biogenesis	FAM96B (CIA2B)		Part of a complex that facilitates Fe–S cluster transfer to specific recipients (*i.e.* GPAT, DYPD)

chromatin marks, thus impeding RNA polymerase II dependent transcription of *FXN*. Using RNase protection assays, Bidichandani et al. showed that the GAA repeat *per se* interferes with *in vitro* transcription of *FXN* in a length-dependent manner [180], consistent with the observed negative correlation between GAA triplet-repeat length and the age at onset of disease. Frataxin is a ubiquitous mitochondrial protein whose exact function is still controversial: however both *in vitro* and *in vivo* studies support the notion that the protein plays a crucial role in Fe–S cluster biogenesis [45,57,181]. FXN deficiency leads to progressive loss of large sensory neurons in the dorsal root ganglia (DRG) and degenerative atrophy of the posterior columns of the spinal cord, resulting in symptoms of progressive ataxia, muscle weakness, and sensory deficits [182]. Non-neurological symptoms include hypertrophic cardiomyopathy and increased incidence of diabetes [183–185]. Cardiac failure is the most common cause of mortality. The condition is characterized by three distinctive and prominent biochemical features: defective maturation of Fe–S cluster enzymes such as aconitase and the respiratory chain complexes [186,187], mitochondrial iron accumulation [108,188–190], and the presence of oxidative stress markers in blood samples [191–193]. Heterozygous individuals who carry the GAA expansion on only one *FXN* allele have no clinical symptoms, though frataxin levels are markedly reduced [194], whereas inactivation of both alleles is embryonically lethal in plants and mice [195,196], and leads to larval stage arrest in *Caenorhabditis elegans* [197], indicating that complete loss of FXN is not compatible with life in higher organisms.

A crucial question regards understanding why specific tissues develop pathology in Friedreich's ataxia, whereas others are spared. Tissues with high levels of frataxin and that are generally highly mitochondria-dependent, such as the kidneys and liver, are not obviously affected in FRDA [164]. The tissue-specific phenotypes of FRDA might arise from the critical requirement of specific isoforms of FXN, which may be severely decreased in the disease. Interestingly, two rare frataxin isoforms have been recently identified [198], and their expression was substantially reduced in the heart (isoform III), and cerebellum (isoform II) of a FRDA patient, suggesting that novel expression patterns may explain some FRDA tissue specific phenotypes.

The fact that frataxin is highly conserved from bacteria to humans led to the development of multiple cellular and animal model systems that have provided insights into the function of frataxin and the pathophysiology of FRDA. Murine FXN is 73% identical to the human protein, thus rendering the mouse a relevant model system in which to investigate the pathogenesis of FRDA [199]. However, only humans and primates have the GAA repeat expansion in the first intron [200], and there is currently no model that is able to reproduce all the symptoms that occur in patients. The combination of data from all the available cell and animal models can help to understand disease processes and develop therapeutic strategies. Several conditional knockout mouse models, based on the Cre–*loxP* system, were generated to investigate the consequences of frataxin depletion in tissues affected in FRDA (particularly nervous system, heart and pancreas). Together, these models reproduce most of the features of the disease, including hypertrophic cardiomyopathy [201], spinocerebellar and sensory ataxia [201–203], and some aspects of the diabetes [204], though they lack the GAA-triplet expansion, which causes incomplete loss of expression in most of the cell types of interest. The resulting phenotypes are therefore partial as they depend on low levels of frataxin in a distinct tissue cell type, in which some cells have full expression, whereas others have completely lost expression. Thus far, two different strategies have been used to generate mouse models that harbor the GAA-repeat expansion, in order to mimic the FRDA condition in which a reduced amount of FXN is present in all tissues: a knock-in approach based on the insertion of a $(GAA)_{230}$ triplet repeat into the first intron of the mouse *FXN* locus (KIKI mice) [205], and the generation of transgenic mice in which the entire human *FXN* gene within a human yeast artificial chromosome (YAC) was inserted onto a mouse *FXN* null background [206]. The various mouse models highlight two main points: on one hand, the conditional knockouts recapitulate important features of FRDA, but they lack the genetic context, whereas the GAA repeat expansion-based knock-in and transgenic models carry a GAA triplet expansion, but they exhibit only a mild phenotype. Cell lines derived from FRDA patients are the most relevant frataxin-deficient *in vitro* models, as they carry the human FRDA mutations. Fibroblasts and lymphocytes from patients are easily accessible but these cell types do not exhibit the strong biochemical phenotype associated with FRDA, despite having reduced frataxin levels [186,207] and higher susceptibility to oxidative stress [193,208]. The most interesting cells to study would be neurons and cardiomyocytes, which are particularly affected in FRDA, but not accessible from patients. To circumvent this problem, primary fibroblasts have been used and reprogrammed into embryonic-like cells, known as induced pluripotent stem cells (iPSCs), that are theoretically able to differentiate into virtually all cell types of the human body [209]. These models explained several biochemical features associated with FRDA, such as the fact that intramitochondrial iron accumulation develops after inactivation of Fe–S-dependent enzymes [181,187]. One mechanism proposed to explain iron accumulation in FRDA patients is related to cytosolic iron deficiency, which would impair biogenesis of extra-mitochondrial Fe–S proteins, particularly cytosolic aconitase, which is converted into the IRE binding form (IRP1), increasing TfR expression and reducing ferritin levels [210]. An additional effect is the stabilization of IRP2, again by cytosolic iron deficiency, that further promotes iron uptake. Moreover, expression of the mitochondrial iron importer, mitoferrin, was found to be increased in the heart of frataxin deficient mice [211]. The resulting mitochondrial iron accumulation may promote significant production of reactive oxygen species (ROS) [187,212,213]. Several pharmacological approaches have been tested to treat the downstream events of the frataxin deficiency, such as mitochondrial iron overload (deferiprone), and oxidative stress (ibedenone), or alternatively to increase frataxin levels (reviewed in [214]). Recently, an extraordinary result and a promising potential therapeutic approach for FRDA patients came from a gene therapy-based strategy. Cardiomyocytes from a conditional mouse model with complete frataxin deletion in cardiac and skeletal muscle were rapidly rescued and remodeled by adeno-associated viral expression of human FXN [215].

5.2. Infantile mitochondrial complex II/III deficiency (IMC23D) caused by a missense mutation in NFS1 on chromosome 20q11.22 (MIM# 603485)

A novel fatal autosomal recessive disease has been identified in three children of healthy third cousins within an Old Order Mennonite community [216], characterized by lactic acidosis, hypotonia, respiratory chain complex II and III deficiencies in muscle, multisystem organ failure and abnormal mitochondrial morphology. The causative mutation led to substitution of Arg72 into Gln in the cysteine desulfurase (NFS1). No NFS1–ISD11 interaction was detected in patient fibroblasts, indicating complete disruption of the multimeric complex that provides sulfur for Fe–S cluster biogenesis [216]. Human NFS1 has been shown to localize either to mitochondria or to the cytosol and nucleus as a result of initiation of translation at alternative in-frame AUGs [51]. Besides its role in Fe–S cluster biogenesis, NFS1 is essential in the cytosol for providing sulfur in the molybdenum cofactor biosynthetic pathway [217], which expands the physiological significance of the cysteine desulfurase, as sulfite oxidase, xanthine oxidoreductase and aldehyde oxidase are essential cytosolic enzymes that require the molybdenum cofactor for their function (reviewed in [218]). Moreover, the cysteine desulfurase is also known to be important in sulfuration of tRNAs [219]. So far, two studies have examined the physiological consequences of cellular NFS1 depletion *via* small interfering RNA-mediated gene silencing approaches [220,221]. Depletion of NFS1 in murine NIH3T3 fibroblasts led to compromised mitochondrial oxidative phosphorylation and reduced activities of several mitochondrial and cytosolic Fe–S enzymes (*i.e.* mitochondrial and cytosolic aconitases and xanthine oxidase) [221]. Similar effects resulted from knock down

of NFS1 in HeLa cells [220]. This represents the first report of NFS1 deficiency in humans, and the early onset of clinical symptoms together with the fatal outcome of the disease stresses the crucial importance of NFS1 for human physiology.

5.3. A homozygous missense mutation in LYRM4 on chromosome 6p25 causes deficiency of multiple respiratory chain complexes (MIM# 615595)

Massively parallel sequencing (MitoExome sequencing) of >1000 mitochondrial genes identified a homozygous mutation in LYRM4/ISD11 (c.203G>T, p.R68L) in a 20-year-old man (P1) from a consanguineous family of Lebanese and Syrian ancestry. The same mutation was also present in his cousin who had a more severe phenotype and died at two months of age (P2). The patients were diagnosed with a mitochondrial disorder characterized by combined oxidative phosphorylation deficiencies in skeletal muscle and liver [49]. In the newborn period, P1 experienced respiratory distress, hypotonia, and severe lactic acidosis. Other features included elevated liver enzymes. He slowly improved with supportive therapy over the following weeks. Laboratory studies on patient liver and muscle samples revealed decreased activities of mitochondrial respiratory chain complexes I, II and III in both patients. P2 also had complex IV deficiency in skeletal muscle. Several Fe–S proteins other than complexes I–III, such as aconitase and ferrochelatase, were also deficient in P1. As cysteine requirements are higher at birth than later in life, Lim and colleagues explained the different clinical outcomes between the two individuals by considering possible differences in the dietary neonatal availability of cysteine between them [49]. The R68L mutation was shown to affect the stability of LYRM4, which was undetectable by western blot in P2 skeletal muscle or in the liver tissue from both patients. The mutant yeast ortholog Isd11^{R71L} failed to rescue growth of an *Isd11*-null strain, consistent with the loss of function effect of the mutation. *In vitro* studies revealed that Isd11^{R71L}, despite binding to NFS1, was unable to activate the cysteine desulfurase enzymatic activity. Overall this finding underscores the crucial role of ISD11 as part of the core complex involved in Fe–S cluster biogenesis, and the phenotype described is consistent with a defect in the early step of Fe–S assembly that affects multiple enzymes.

5.4. ISCU myopathy, also known as hereditary myopathy with lactic acidosis or as Swedish myopathy, caused by homozygous or compound heterozygous mutations in the ISCU gene on chromosome 12q24 (MIM# 255125)

Hereditary myopathy with lactic acidosis (HML), also known as Swedish myopathy with exercise intolerance [222,223], was first described in the 1960s and is an autosomal recessive muscular disorder characterized by childhood onset of exercise intolerance with cramping, dyspnea, palpitations and muscle weakness. Biochemical features of the disease include lactic acidosis and, rarely, rhabdomyolysis and myoglobinuria [222,224,225], with impaired muscle oxidative phosphorylation, deficient succinate dehydrogenase [224,226] and aconitase [227,228] activities, and the presence of iron aggregates in mitochondria [227,228]. Further biochemical characterization of mitochondria from HML patients [228] showed that, in addition to severe deficiencies of complex II and aconitase, complex III activity decreased to 37% of normal, and immunoblot analysis of complex I revealed reduced levels of several subunits, suggesting a generalized abnormality of the synthesis, import, processing or assembly of a group of Fe–S cluster containing enzymes. In 2008, two groups identified the disease causing mutation in patients from northern Sweden with HLM within a common region of homozygosity on chromosome 12q24.1 [227,229]. The point mutation was an intronic G to C transversion (7044G-C) in the *ISCU* gene, which strengthened a weak splice acceptor site and resulted in the retention of a 100 base pair (bp) fragment of intron 5 in the mature mRNA. The aberrantly spliced *ISCU* mRNA encoded the wrong C-terminal sequence, which ends with a premature stop codon, leading to translation of a truncated protein with a markedly reduced half-life [230]. Given the role of ISCU as the primary scaffold for Fe–S cluster biogenesis, the mutation leads to adverse effects on Fe–S protein biogenesis and intracellular iron homeostasis. In fifteen affected members from nine families [222,223], the same mutation in intron 5 of *ISCU* was independently identified [229]. Two brothers with myopathy, lactic acidosis and cardiac involvement who exhibited a more severe phenotype than other reported patients were found to be compound heterozygotes for the common intronic mutation (7044G-C) and for a c.149G>A missense mutation in exon 3 of the second *ISCU* allele, changing a highly conserved glycine residue into glutamate (G50E) [231]. A recent biochemical characterization of ISCU^{G50E} highlighted that the mutation interferes with the ability of the scaffold protein to interact with the sulfur donor NFS1 and with the cochaperone HSC20, thus drastically impairing the rate of Fe–S cluster synthesis [232]. Lack of ISCU in muscle tissue from ISCU myopathy patients elicits activation of a systemic metabolic response leading to increased plasma levels of the starvation response hormone FGF21 (fibroblast growth factor 21) [233]. Interestingly, the incorrect splicing of the *ISCU* mRNA is more pronounced in muscle than in other tissues, resulting in a muscle-specific phenotype. Moreover, the differentiation level of muscle fibers was shown to enhance aberrant splicing of ISCU, as the muscle-specific transcription factor MyoD1 decreased the amount of normal ISCU transcript in patient myoblasts [233]. Several studies were carried out to understand the molecular mechanisms that may contribute to the tissue specific phenotype of the disease, and five nuclear factors were found to interact with the sequence harboring the mutation [234], including three splicing factors, SFRS14, RBM39, and PTBP1, and two additional RNA binding factors, matrin 3 (MATR3) and IGF2BP1. IGF2BP1 showed a preference for the mutant sequence, whereas the other factors had similar affinity for both sequences. PTBP1 was found to repress the defective splicing of ISCU, whereas IGF2BP1 and RBM39 shifted the splicing ratio toward the incorrect form. Differential expression of these factors in a tissue specific manner could explain the severity of the phenotype in the skeletal muscle of ISCU myopathy patients.

A first report in 2009 demonstrated the ability of an antisense oligonucleotide (AO), which specifically targeted the activated cryptic splice sites in the *ISCU* gene, to restore the correct reading frame in cultured fibroblasts derived from patients with the homozygous mutation. The restoration was stable, with the correctly spliced mRNA remaining the dominant species after 21 days [235]. More recently two different antisense oligonucleotides were tested for their ability to correct the aberrant splicing in muscle-derived primary cells from ISCU myopathy patients. The oligonucleotides were based on the phosphorothioate chemistry and they targeted the splice acceptor or splice donor site of the disease-associated pseudoexon [236]. Opposite effects were observed with the two AOs, as the acceptor site antisense oligonucleotide effectively redirected splicing toward the normal transcript in cultured muscle fibroblasts, whereas the donor site oligonucleotide promoted intron inclusion in both patient and control cells. Since only a shift in balance between abnormal and normal splice variants, and not complete normalization of splicing, seems to be necessary to alleviate the biochemical defects in HML, the AO treatment appears to be a promising therapeutic strategy in the ISCU myopathy. However, it highlights the importance of optimizing the sites targeted by the antisense oligonucleotides to ensure that proper pre-mRNA splicing takes place.

5.5. Mitochondrial encephalopathy with deficiency in NUBPL (IND1) (MIM 613621)

Deficiency of respiratory chain complex I (NADH: ubiquinone oxidoreductase) is the most common causative factor underlying mitochondrial disorders, accounting for approximately a third of all cases of OXPHOS disorders [100,237]. Complex I is the largest enzyme of the

mitochondrial respiratory chain, and its characteristic L-shaped structure was initially revealed by electron microscopy. X-ray crystallographic studies of the complexes isolated from *Thermus thermophilus*, *Y. lipolitica*, and *E. coli* subsequently provided further structural details [238–242]. Ongoing efforts have been made to determine the position of the different subunits in the mammalian enzyme by crystallizing purified complex I from bovine heart. Human complex I is very similar to the bovine complex and consists of 45 different subunits [243], 14 of which constitute the core catalytic subunits and are conserved in all species that have complex I, including prokaryotes [244]. Seven of these core subunits are hydrophobic and mitochondrially encoded, and the other seven are hydrophilic and encoded by the nuclear genome. The remaining 31 subunits are often referred to as accessory or supernumerary subunits. Central to complex I enzymatic activity is a chain of eight Fe–S clusters in the peripheral arm that facilitates transfer of single electrons from NADH to ubiquinone. The energy generated by the electron transfer is transduced to the membrane arm, which then pumps protons into the intermembrane space to generate the electrochemical gradient utilized for ATP synthesis [240]. Sequencing of the known 45 genes encoding structural subunits of complex I offered a genetic explanation for about 50% of affected individuals [146], thus suggesting that additional factors that are not integral to the mature complex are required for effective complex I assembly and function [100,245,246]. Ind1 has been identified and characterized as an assembly factor for complex I in the model organism *Y. lipolitica* [144]. Ind1 was proposed to be essential for the assembly of Fe–S clusters for complex I on the basis of several observations: it has sequence similarities to Nbp35 and Cfd1, which are scaffold proteins for cytosolic Fe–S cluster biogenesis, it is targeted to mitochondria and is able to bind a labile Fe–S cluster *in vitro*, and its knock down had adverse effects on complex I activity and assembly [144]. The human ortholog, NUBPL (nucleotide-binding protein-like protein) or IND1, was similarly proposed to be crucial for delivery of Fe–S clusters to complex I [145]. However, recent studies in *A. thaliana* demonstrated a primary role of IND1 (INDH) in mitochondrial translation, which secondarily affects complex I assembly because seven of its core subunits are mitochondrially encoded [147]. Nevertheless, NUBPL mutations are known to cause defects in complex I activity and assembly associated with mitochondrial encephalopathy [146,247]. The c.815-27T>C branch-site mutation in *NUBPL* affects mRNA splicing [146,248], and it was identified in seven unrelated individuals with mitochondrial syndromes. High-throughput screen of a cohort of 103 patients with complex I deficiency led to identification of a second missense mutation in exon 2 in the *NUBPL* gene [146], c.166G>A, which causes the substitution of Gly56 into Arg [248]. Most of the patients are compound heterozygotes with one null allele and the other carrying the branch-site mutation (c.815-27T>C) in combination with a c.166G>A missense mutation [249,250]. All the patients exhibit a characteristic leukoencephalopathic pattern on brain MRI, confluent or multifocal cerebral white matter lesions, along with abnormalities and swelling of the corpus callosum. Moreover, patients developed motor problems due to ataxia in the first years of life, whereas other features were more variable, as some patients showed continuous regression whereas others manifested only episodic regression with differences in cognitive capabilities ranging from normal to significantly deficient. In a German patient, two mutations were identified in the *NUBPL* gene [250]: a 10-bp insertion in exon 8 on one allele, resulting in premature termination (Glu223AlafsTer4) and the combination of c.166G>A/c.815-27T>C on the other allele. Different missense mutations in NUBPL (D105Y, L193F) were also found in two Canadian siblings and in a patient from the Netherlands, in combination with c.166G>A/c.815-27T>C [250]. Finally, a G to A transition in intron 8, predicted to cause skipping of exon 8 was identified in an American patient, who harbored the c.166G>A/c.815-27T>C mutations on the second allele [250].

5.6. Mitochondrial muscle myopathy with deficiency of ferredoxin 2 (FDX1L)

The human genome contains two homologous ferredoxins, *FDX1*, located on chromosome 11q22 and highly expressed in adrenal cortex and medulla, and the ubiquitous *FDX1L*, renamed *FDX2*, on chromosome 19p13.2 [63]. Both FDXs are involved in Fe–S cluster biogenesis, together with ferredoxin reductase (FDXR), providing reducing equivalents [32]. FDX1 was also found to be essential for the synthesis of various steroid hormones in adrenal glands [63]. *FDX1L* encodes a 183 amino acid [Fe_2–S_2] mitochondrial protein. A homozygous mutation c.1A>T was recently identified in the *FDX1L* gene, which disrupts the ATG initiation translation site, resulting in severe reduction of FDX2 levels in the patient muscle and fibroblasts mitochondria [251]. The patient presented with an adolescent onset proximal muscle myopathy associated with recurrent episodes of myoglobinuria and lactic acidosis. The biochemical features of the disease included severe reductions in the activities of mitochondrial aconitase (25% of normal) and of the Fe–S dependent respiratory chain complexes I to III in the patient's skeletal muscle (6–36% of normal), with complex II being the most severely affected, whereas citrate synthase activity was normal, consistent with the crucial role of FDX2 as a core component of the Fe–S cluster assembly machinery. Elevated lactate, ketones and Krebs cycle metabolites detected in the urine, together with low pyruvate dehydrogenase complex (PDHc) activity in muscle homogenate were indicative of a generalized metabolic remodeling caused by impaired Fe–S cluster biogenesis for several mitochondrial enzymes, including lipoic acid synthase.

5.7. Lipoic acid defects due to mutations in Fe–S cluster biogenesis components (NFU1, BOLA3, IBA57, and GLRX5)

Lipoic acid (LA) is a covalently bound cofactor essential for five redox reactions in humans: in four 2-oxoacid dehydrogenases and in the glycine cleavage system (GCS) (reviewed in [252]). Two enzymes are essential for energy metabolism (α-ketoglutarate dehydrogenase α-KGDH, and pyruvate dehydrogenase PDH), and three are involved in amino acid metabolism pathways (branched-chain ketoacid dehydrogenase BCKDH, 2-oxoadipate dehydrogenase, and the GCS). LA synthesis has been extensively investigated in *S. cerevisiae* [253], and for most but not all of the involved enzymatic reactions, orthologues have been identified in humans. LA is synthesized in mitochondria by a series of reactions involving transfer of an octanoyl group to a first acceptor protein (H protein of the GCS), lipoate synthesis by insertion of two sulfhydryl groups into the side chain of the bound octanoic acid, and final transfer of newly synthesized lipoate from the H protein to the E2 subunits of 2-oxoacid dehydrogenases. The octanoyl moiety attached to a conserved lysine of the H protein of the GCS [254] is converted into lipoate by sulfurylation at positions 6 and 8 catalyzed by the radical S-adenosylmethionine (SAM) enzyme lipoic acid synthase (LIAS) [255]. LIAS has two [Fe_4–S_4] clusters, which are essential for the enzymatic reaction as one of them provides sulfur for sulfurylation of octanoate to lipoate, during catalysis [252]. Lipoic acid defects were found in four syndromes caused by mutations in *NFU1* (MMDS1, multiple mitochondrial dysfunctions syndrome 1 [133,135,136]), *BOLA3* (MMDS2, multiple mitochondrial dysfunctions syndrome 2 [133,134,256]), *IBA57* (MMDS3, multiple mitochondrial dysfunctions syndrome 3 [36]), and *GLRX5* (non ketotic hyperglycinemia [256]). Clinical features of individuals affected by the multiple mitochondrial dysfunctions syndromes, which is often fatal at perinatal stages, include hypotonia, respiratory insufficiency, encephalopathy, myopathy, brain malformation, and neurological regression. Patients have metabolic acidosis with elevated blood lactate levels, hyperglycinemia, compromised mitochondrial respiration, and reduced activities of the lipoic acid dependent enzymes PDH, α-KGDH, BCKDH and GCS.

5.8. Multiple mitochondrial dysfunctions syndrome 1 (MMDS1) is caused by mutations in NFU1 (MIM# 605711)

Initially reported in 2001 by Seyda et al. in three siblings of Mexican origins [257], MMDS1 is a severe autosomal recessive disorder of systemic energy metabolism, resulting in weakness, respiratory failure, lack of neurologic development, lactic acidosis and early death. The underlying genetic defect of MMDS1 was later identified by Cameron et al. [133] in the *NFU1* gene: a homozygous missense mutation c.545G>A near the splice donor site of exon 6 caused aberrant splicing of *NFU1* mRNA and no mature protein could be detected in fibroblasts from the patients. Biochemical analysis revealed severe deficiencies of pyruvate dehydrogenase complex (PDHc) and α-ketoglutarate dehydrogenase complex (α-KGDH), due to reduced lipoylation of the E2 subunits of these enzymes, and decreased activities of respiratory chain complexes I, II and III, whereas aconitase activity was normal. High glycine, leucine, valine and isoleucine levels were detected in the urine samples from the patients indicating abnormalities of the GCS and branched-chain α-ketoacid dehydrogenase, both of which depend on covalently attached lipoate. In 9 patients from unrelated Spanish families with fatal infantile encephalopathy, hyperglycinemia, pulmonary hypertension, lactic acidosis and impaired PDHc activity, Navarro-Sastre et al. [135] identified a homozygous mutation in the *NFU1* gene, causing the missense mutation G208C, which was shown to impair NFU1 function. MMDS1 results from compromised biogenesis of LIAS, which has been proposed to acquire its Fe–S clusters from NFU1.

5.9. Multiple mitochondrial dysfunctions syndrome 2 (MMDS2) is caused by mutations in BOLA3 (MIM# 614299)

The clinical and biochemical phenotypes of MMDS2 caused by mutations in *BOLA3* closely resemble the condition determined by NFU1 deficiency, with a severe epileptic encephalopathy and dilated cardiomyopathy, elevated lactate and glycine levels, reduced mitochondrial respiration and low PDH, α-KGDH and GCS activities reported in at least six patients identified so far [133,134,256,257]. Three different types of missense mutations in *BOLA3* were found to be responsible for the disease: a homozygous single base pair duplication (c.123dupA) leading to a frameshift and a premature stop codon (p.Glu42Argfs*13) [133], a c.136C>T point mutation in exon 2 of the *BOLA3* gene producing a truncated protein (p.R46* stop codon) [256], causing the less severe phenotype reported, and a homozygous mutation on exon 3 of the *BOLA3* gene, resulting in the substitution of Ile67 into Asn [134]. Because of the similar phenotypes of MMDS1 and MMDS2, BOLA3 has been proposed to function in concert with NFU1 for biogenesis of LIAS [133]. However, this hypothesis needs to be supported by further experimental evidence.

5.10. Multiple mitochondrial dysfunctions syndrome 3 (MMDS3) is caused by mutations in IBA57 (MIM# 615330)

Bolar et al. [36] reported the case of two siblings, born from consanguineous Moroccan parents, with a lethal encephalomyopathy and myopathy resulting from mitochondrial dysfunction. Laboratory studies showed severe lactic acidosis and increased glycine in cerebrospinal fluid (CSF). Both infants died soon after birth. The severe phenotype was caused by a homozygous missense mutation in the *IBA57* gene, resulting in a Gln314Pro substitution in a non conserved region of the protein. Biochemical characterization of $IBA57^{Q314P}$ in HeLa cells showed that the missense mutation diminished the activity of IBA57, and the mutant protein was particularly prone to proteolytic degradation. Although the exact molecular function of IBA57 is not known, experimental evidences obtained in cell lines and in the patients have suggested that it is involved in the biogenesis of $[Fe_4–S_4]$ mitochondrial proteins [36,143].

5.11. Mutations in GLRX5 cause sideroblastic anemia or variant non-ketotic hyperglycinemia

A homozygous 294A>G transition in the third nucleotide of the last codon of *GLRX5* exon 1 gene was identified in 2007 in a southern Italian man with autosomal recessive pyridoxine-refractory siderobastic anemia [109]. The mutation was shown by RT-PCR to interfere with correct splicing. Sideroblastic anemias (SAs) are a group of heterogeneous congenital and acquired disorders that have common features of mitochondrial iron accumulation in bone marrow erythroid precursors (ringed sideroblasts), ineffective erythropoiesis, increased levels of tissue iron and varying amounts of hypochromic erythrocytes in the peripheral blood [258,259]. Congenital sideroblastic anemia is a rare disease caused by mutations of genes involved in heme biosynthesis, Fe–S cluster biogenesis, and mitochondrial protein synthesis (Pearson's marrow-pancreas syndrome or PMPS). The most common form is the X-linked sideroblastic anemia, caused by mutations in the erythroid-specific δ-aminolevulinate synthase (ALAS2), the first enzyme of the heme biosynthetic pathway in erythroid cells [259,260]. The human genome contains two *ALAS* genes: the *ALAS2* transcript has an IRE in its 5′-UTR and is specifically expressed in erythroid cells, whereas the *ALAS1* transcript lacks an IRE and is ubiquitously expressed. Other mutations known to cause sideroblastic anemias are mitochondrial DNA deletions [261], or mutations in the erythroid specific mitochondrial transporter (SLC25A38) [262], the adenosine triphosphate (ATP) binding cassette B7 (ABCB7) [263], the thiamine transporter SLC19A2 [264], the tRNA-modifying enzyme pseudouridine synthase (PUS1) [265], the mitochondrial tyrosyl-tRNA synthase (YARS2) [266], and the glycyl-tRNA synthetase (GARS) [267]. An animal model for studies of SA caused by loss of glutaredoxin 5 is zebrafish *shiraz*, a pale mutant in which blood cells are severely hypochromic, definitive hematopoiesis is defective, and the condition is lethal between day 7 and 10 after fertilization [103]. The *shiraz* phenotype is caused by a large deletion encompassing the *GLRX5* gene. Loss of GLRX5 compromises Fe–S cluster biogenesis, and causes activation of IRE binding activity of IRP1 (IRP2 was found not to have a role in *shiraz*), which ultimately leads to anemia due to IRP-mediated inhibition of *ALAS2* translation [103]. Loss of its Fe–S cluster converts IRP1 from cytosolic aconitase (ACO1) into the IRE-binding form. IREs are present in either the 5′ or 3′ terminal UTR of several gene transcripts, most of which encode proteins involved in iron metabolism, that are respectively repressed or stabilized by IRP1 binding. The transcript of *ALAS2*, which catalyzes the first step of heme synthesis in mitochondria of erythroblasts, contains a 5′-IRE, and its translation is repressed by IRP1 binding. Ferrochelatase contains a labile $[Fe_2–S_2]$ cluster that is required for its function and stability [268]. Therefore, activities of the first and last steps of heme biosynthesis in erythroid cells are governed by the Fe–S cluster biogenesis status in cells, either by determining activity of IRP1 or by directly contributing to activity/stability of a Fe–S enzyme [108]. Overexpression of ALAS2 without the 5′-IRE rescued *shiraz* embryos, as also did knockdown of IRP1, whereas overexpression of the 5′-IRE isoform of ALAS2 did not [103]. The GLRX5 patient was an anemic middle-aged man when he started developing severe symptoms, including microcytic hypochromic anemia, type II diabetes, cirrhosis, and liver iron overload [109]. Patient cells showed decreased GLRX5 expression, low levels of H-ferritin and ferrochelatase and high TfR, consistent with activation of IRE-binding activity of IRP1. Loss of GLRX5 resulted in mitochondrial iron overload and concomitant cytosolic iron depletion with subsequent stabilization of IRP2, which started a vicious cycle enhancing repression of ferritin and ALAS2 in erythroblasts and increasing mitochondrial iron [108]. Anemia was partially reversed by iron chelation, perhaps by promoting a redistribution of iron to the cytosol, which might lead to IRP2 degradation, thus improving heme synthesis and anemia [109]. The phenotypes caused by loss of GLRX5 underscore the causal link between Fe–S cluster biogenesis and heme synthesis, with IRP1 being a major player in the process [12,13,269,270].

A completely different phenotype has been recently identified in three individuals harboring different mutations in the *GLRX5* gene [256], two of whom shared a Lebanese origin and a homozygous deletion c.151_153delAAG (p.K51del) in *GLRX5* exon 1. The third patient of Chinese descent had the same deletion on the maternal allele and an 8 bp insertion c.82_83insGCGTGCGC (p.G28Gfs*25), resulting in a premature stop codon at amino acid 52, 25 amino acids downstream, on the paternal allele. The deleted Lys51 is highly conserved from human to *C. elegans*, and is predicted to be deleterious *in silico*. It is located on the surface, between the first α-helix and the first β-sheet of GLRX5, and it's close to K59, which interacts directly with the Fe–S cluster at the active site [256]. The patients were diagnosed with non-ketotic hyperglycinemia characterized by specific deficiencies in lipoate metabolism, pointing to improper biogenesis of LIAS. Glycine accumulates in blood and brain due to compromised activity of the GCS and contributes to the neurodegenerative phenotype [271]. Interestingly, no defect in Fe–S enzymes other than LIAS has been identified in any of the three patients, iron metabolism was found to be normal, and their phenotype was completely different from the patient affected by SA, who conversely did not show any neurological symptoms.

5.12. X-linked sideroblastic anemia with cerebellar ataxia (XLSA/A) due to mutations in ABCB7

First isolated in 1997 from a murine macrophage cDNA library [272], the ATP-binding cassette (ABC) transporter ABCB7 was mapped to the X chromosome both in mice and humans [272]. Mutations in *ABCB7* cause X-linked sideroblastic anemia with cerebellar ataxia (XLSA/A), a recessive disorder characterized by an infantile to early childhood onset of non-progressive cerebellar ataxia and mild anemia with hypochromia and microcytosis. A distinguishing feature of the disease is the presence of iron-loaded mitochondria (sideroblasts), and defects in heme metabolism with protoporphyrin IX accumulation [273–275]. Defects in ABCB7 have also been proposed to mediate ineffective erythropoiesis in refractory anemia with ring sideroblasts (RARS) [276,277], a myelodysplastic syndrome (MDS) characterized by mitochondrial ferritin (FTMT) accumulation, and in the refractory anemia with ring sideroblasts and isodicentric (X) (q13) chromosome [278]. It seems that the anemia and the ataxia observed in XLSA/A are related to altered iron homeostasis which leads to mitochondrial iron accumulation in developing red blood cells and in neural cells [279,280].

ABCB7 is the human ortholog of yeast Atm1. Atm1 has been recently crystallized in its nucleotide-free and glutathione-bound form [281], and it localizes to the inner mitochondrial membrane [282] with the nucleotide binding domain (NBD) facing the matrix: therefore it is thought to function as an exporter. Upon Atm1 depletion, mitochondria accumulate iron [153,154,283,284]. Depletion of ABCB7 orthologs in plants, mice and HeLa cells results in loss of cytosolic aconitase activity, mitochondrial iron accumulation and increased sensitivity to oxidative stress, whereas no significant changes in activities of mitochondrial Fe–S enzymes (*i.e.* ACO2 or SDH) have been reported [153,154,280,285,286]. It has been proposed that Atm1 exports a sulfur-containing compound necessary for biogenesis of Fe–S clusters in the cytosol [107,153,156]. However this hypothesis has been recently questioned because genetic manipulations, which were carried out to inactivate *ATM1*, also introduced *LEU2* into a *LEU2*-null strain (Δ*LEU2*), which unexpectedly led to transcriptional repression of *LEU1* relative to the control strain [287]. Leu1 is the isopropyl malate isomerase, a cytosolic Fe–S protein that has been used to assess cytosolic Fe–S cluster biogenesis. Because Leu1 activity was low in Atm1 null strains, this was misinterpreted as evidence that Atm1 contributed to export of a component essential to cytosolic Fe–S cluster biogenesis [287]. Loss of Atm1 can be partially compensated by the overexpression of an Atm1 homologue, Mdl1, which exports peptides derived from the degradation of mitochondrial inner membrane proteins to the intermembrane space and cytosol [288,289]. Orthologs of yeast Atm1 are found in virtually all eukaryotes and some bacteria [290], and the ABC transporter ATM3 in *A. thaliana*, which can functionally complement a yeast Δ*ATM1* strain [291], was found to be essential for the export of the cyclic pyranopterin monophosphate (cPMP) intermediate from mitochondria to the cytosol for the biogenesis of the molybdenum cofactor [292]. It has been therefore proposed that ATM3 transports two distinct compounds or a single molecule required for both Fe–S and molybdenum cofactor biogenesis in the cytosol [285]. Interestingly, neither *ATM1* nor *ATM3* null strains are lethal, suggesting that there might be some redundancy between different ABC transporters. Atm1 binds glutathione (GSH) with high affinity during the purification steps carried out in *E. coli* [281]. GSH stimulates the ATPase activity of Atm1, and Atm1- deficient mitochondria accumulate glutathione *in vivo* [286,293]. However, GSH itself cannot be the physiological substrate because it is synthesized in the cytosol, where the main cellular pool resides, though it could be part of the exported moiety [281]. Interestingly, all the residues that coordinate GSH were found to be conserved in Atm1-like proteins, including human ABCB7 and the bacterial orthologs [281]. One of these residues, D398, corresponds to E433 on the human ABCB7, and it is mutated into lysine in patients with XLSA/A, showing the physiological relevance of the substrate-binding pocket of the transporter [274]. Three of the XLSA/A patient mutations are located in the membrane domain either on the matrix [294] or the intermembrane space sides [273–275], and they are highly conserved, suggesting their critical importance for ABCB7 function [281].

5.13. Variant erythropoietic protoporphyria caused by abnormal expression of mitoferrin 1

Erythropoietic protoporphyria (EPP) is an inborn metabolic disorder characterized by accumulation of protoporphyrin in red cells and in the skin, where it causes photosensitivity [295–297]. The most severe clinical feature is hepatobiliary disease due to protoporphyrin-induced damage to liver cells [298]. Deficiency of the Fe–S enzyme ferrochelatase (FECH), which catalyzes the insertion of ferrous iron into protoporphyrin to form heme, is the most common cause of EPP [299–301]. A variant form of EPP has been linked to aberrant splicing of *MFRN1* mRNA, which encodes the erythroblastic specific solute carrier mitoferrin 1 (MFRN1, SLC25A37), the major importer of iron into mitochondria in erythroblastic cells [302]. MFRN1 has a paralog, MFRN2, which is mostly expressed in non-erythroid cells [303]. Fine regulation of iron import into mitochondria is essential for heme biosynthesis, hemoglobin production and Fe–S cluster biogenesis during red cell development. Shaw et al. described a zebrafish mutant, frascati (frs), that had profound hypochromic anemia and erythroid maturation arrest due to defects in mitochondrial iron uptake [302]. Through positional cloning, they found that the gene mutated in the frs mutant was a member of the vertebrate mitochondrial solute carrier family (SLC25) which was named mitoferrin (mfrn). Mitoferrin 1 is highly expressed in fetal and adult hematopoietic tissues of zebrafish and mouse. Erythroblasts generated from murine embryonic stem cells null for Mfrn showed maturation arrest with severely impaired incorporation of ^{55}Fe into heme. Disruption of the yeast mfrn orthologs, *MRS3* and *MRS4*, caused defects in iron metabolism and in mitochondrial Fe–S cluster biogenesis [304–306]. MFRN1 was found to be part of an oligomeric complex containing also FECH and ABCB10, which was proposed to integrate mitochondrial iron import with heme synthesis [307]. Therefore, defective MFRN1 could decrease transport of iron to FECH, or impair formation of the $[Fe_2–S_2]$ cluster essential for human FECH activity [308], and for the stability of the protein [268]. Six out of seven patients affected by EPP were found to have an insertion of a 477 bp segment in the *MFRN1* gene, due to activation of a cryptic donor splice site (GT) in intron 2, which causes a nonsense codon (TAG) at amino acid 156 [309]. Importantly, five of the affected individuals harbored also defects in the genes of ferrochelatase and *ALAS2*, which complicates the interpretation of the phenotype associated with accumulation of

porphyrin. Loss of MFRN1 in zebrafish and mice leads to profound anemia, but mutant animals do not show signs of protoporphyria. The mechanism that prevents protorphyria in *MFRN1* knock out animals relies on the regulatory role played by IRP1. IRP1 attenuates protoporphyrin biosynthesis by binding to the 5′ IRE of *ALAS2* mRNA, inhibiting its translation and preventing protoporphyrin accumulation. IRP1 deficiency or ectopic expression of *ALAS2* harboring a mutated IRE that can't bind IRP1 is responsible for protoporphyria [310]. In patients with EPP, mis-splicing of *MFRN1* mRNA is particularly frequent, although the underlying mechanism has not been determined. The majority of these mRNAs are degraded by nonsense-mediated decay, whereas the remaining transcripts encode a truncated protein that is not properly targeted to mitochondria. The net result is a significant decrease in the amount of functional MFRN1, which is in turn responsible for reduced import of iron into mitochondria with adverse effects on Fe–S cluster biogenesis and heme synthesis.

6. Mitochondrial iron homeostasis and Fe–S cluster biogenesis

It is known that defects in the basic pathway of Fe–S cluster biogenesis lead to mitochondrial iron overload and cytosolic iron depletion in mammalian cells [30,31,311]. The regulatory mechanisms of this crucial pathway are different in yeast and mammals, but a dynamic, fine balance between mitochondrial iron requirements for Fe–S cluster biogenesis, heme synthesis and cellular iron regulation appears to be a key signal that supports normal homeostasis. In yeast, iron uptake and intracellular distribution mainly rely on a transcriptional regulatory mechanism that involves Aft1 and Aft2 [312], two transcriptional factors which translocate to the nucleus under low iron conditions to activate genes of the iron regulon to control iron uptake and intracellular distribution. Yeast strains harboring deletions of the cysteine desulfurase Nfs1 have a >30-fold increase in mitochondrial iron levels compared to wild type strains [153,313]. Similarly, depletion of components of the Fe–S cluster biogenesis machinery, such as Yfh1 [314], Isu1, Isu2 [315,316], Nfu1, Isa1, Isa2 [139], Ssq1 [317], Jac1 [90], Yah1 [318] impairs Fe–S biogenesis and causes mitochondrial iron overload [319], in the form of iron (III) phosphate nanoparticles [320,321]. Aft1 activates genes of the iron regulon in yeast strains with mutations in Nfs1 and Ssq1 [313], perhaps by responding to a signal from an Fe–S protein or to the altered mitochondrial utilization of iron for the biogenesis of Fe–S clusters [322,323]. In mammalian cells, mitochondrial iron overload occurs concomitantly with cytosolic iron depletion as judged by activation of IRPs [50,68,210], and it is a characteristic feature of the heart and neurons of FRDA patients [324], the skeletal muscle of individuals with ISCU myopathy [227], the precursors of red blood cells in a patient with SA caused by deficiency of GLRX5 [108], and in individuals affected by X-linked sideroblastic anemia and ataxia caused by mutations in ABCB7 [273]. Both in yeast and in humans, mitochondrial iron overload can be readily reversed by restoration of the missing Fe–S cluster biogenesis protein. Several hypotheses have been proposed to explain the intimate connection between Fe–S cluster biogenesis and iron homeostasis. It has been suggested that iron accumulates in mitochondria when Fe–S cluster biogenesis is compromised because it cannot be incorporated into Fe–S clusters destined to ligate to mitochondrial proteins or to be exported in the form of a precursor in order to support cytosolic Fe–S cluster biogenesis [153]. Later on, this hypothesis was dismissed, as it was suggested that ABCB7 (Atm1 in yeast) exports a sulfur-containing compound, which no longer provides an explanation for mitochondrial iron overload [107,156]. One possibility could be that an Fe–S protein is directly or indirectly involved in the sensing of iron levels in mitochondria, so that compromised Fe–S biogenesis might be registered as insufficient provision of iron to the mitochondrial compartment. This could result in a feedback regulation and activation of a response that includes the increased delivery of iron to mitochondria and concomitant depletion of the cytosolic iron pool, which finally engages the cell in a vicious cycle in which increased cellular iron uptake further exacerbates mitochondrial iron overload [313,325]. In support of this idea, transcriptional expression of the mitochondrial iron importer mitoferrin is increased in frataxin-deficient mouse hearts [211], and in muscle biopsies from the ISCU myopathy patients [233]. An attractive possibility is that a molecule exported from mitochondria acts as a signal that drives iron import. Active export of peptides from the mitochondrial matrix to the intermembrane space in *S. cerevisiae* is accomplished by the ABC transporter Mdl1, which was proposed to be crucial for yet-to-be-determined signal transduction pathways in yeast [288]. Several cellular pathways, such as the stress of protein misfolding in the mitochondrial matrix, have been dissected, and they were found to rely on export of signaling molecules, which activate nuclear-encoded mitochondrial genes [326–328]. Mdl1 is homologous to Atm1 and it can partially compensate for *ATM1* loss when overexpressed. The Atm1/ABCB7 substrate is so far unknown. It might be interesting to investigate the nature of the molecule that is exported by Atm1/ABCB7, as this could help the understanding of the regulatory networks that are key to the control of mitochondrial iron homeostasis.

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